

In response to the March 12, 2004 Office Action, please amend the above application as follows:

Amendments To The Specification

(1) Please replace the underlined subsection **heading at page 16, line 29** with the following amended heading:

Inactivation of Fungal Glycosylation Enzymes such as 1,6- mannosyltransferase

(2) Please replace the paragraph spanning **page 17, line 16 to page 18, line 21** with the following amended paragraph:

Genes that are involved in this process have been identified in *Pichia pastoris* and by creating mutations in these genes one is able to reduce the production of “undesirable” glycoforms. Such genes can be identified by homology to existing mannosyltransferases (e.g. OCH1, MNN4, MNN6, MNN1), found in other lower eukaryotes such as *C. albicans*, *Pichia angusta* or *S.cerevisiae* or by mutagenizing the host strain and selecting for a phenotype with reduced mannosylation. Based on homologies amongst known mannosyltransferases and mannosylphosphate transferases, one may either design PCR primers, examples of which are shown in Table 2, or use genes or gene fragments encoding such enzymes as probes to identify homologues in DNA libraries of the target organism. Alternatively, one may be able to complement particular phenotypes in related organisms. For example, in order to obtain the gene or genes encoding 1,6-mannosyltransferase activity in *P. pastoris*, one would carry out the following steps. OCH1

mutants of *S. cerevisiae* are temperature sensitive and are slow growers at elevated temperatures. One can thus identify functional homologues of OCH1 in *P.pastoris* by complementing an OCH1 mutant of *S.cerevisiae* with a *P.pastoris* DNA or cDNA library. Such mutants of *S.cerevisiae* may be found e.g., see the *Saccharomyces* genome link at the Stanford University website and are commercially available. Mutants that display a normal growth phenotype at elevated temperature, after having been transformed with a *P.pastoris* DNA library, are likely to carry an OCH1 homologue of *P.pastoris*. Such a library can be created by partially digesting chromosomal DNA of *P.pastoris* with a suitable restriction enzyme and after inactivating the restriction enzyme ligating the digested DNA into a suitable vector, which has been digested with a compatible restriction enzyme. Suitable vectors are pRS314, a low copy (CEN6/ARS4) plasmid based on pBluescript containing the Trp1 marker (Sikorski, R. S., and Hieter, P., 1989, *Genetics* 122, pg 19-27) or pFL44S, a high copy (2 μ) plasmid based on a modified pUC19 containing the URA3 marker (Bonneaud, N., et al., 1991, *Yeast* 7, pg. 609-615). Such vectors are commonly used by academic researchers or similar vectors are available from a number of different vendors such as Invitrogen (Carlsbad, CA), Pharmacia (Piscataway, NJ), New England Biolabs (Beverly, MA). Examples are pYES/GS, 2 μ origin of replication based yeast expression plasmid from Invitrogen, or Yep24 cloning vehicle from New England Biolabs. After ligation of the chromosomal DNA and the vector one may transform the DNA library into strain of *S.cerevisiae* with a specific mutation and select for the correction of the corresponding phenotype. After sub-cloning and sequencing the DNA fragment that is able to restore the wild-type phenotype, one may use this fragment to eliminate the activity of the gene product encoded by OCH1 in *P. pastoris*.

(3) Please replace the paragraph spanning **page 25, line 22 to page 26, line 6** with the following amended paragraph:

Since the ultimate goal of this genetic engineering effort is a robust protein production strain that is able to perform well in an industrial fermentation process, the integration of multiple genes into the fungal chromosome involves careful planing. The engineered strain will most likely have to be transformed with a range of different genes, and these genes will have to be transformed in a stable fashion to ensure that the desired activity is maintained throughout the fermentation process. Any combination of the following enzyme activities will have to be engineered into the fungal protein expression host: sialyltransferases, mannosidases, fucosyltransferases, galactosyltransferases, glucosyltransferases, GlcNAc transferases, ER and Golgi specific transporters (e.g. syn and antiport transporters for UDP-galactose and other precursors), other enzymes involved in the processing of oligosaccharides, and enzymes involved in the synthesis of activated oligosaccharide precursors such as UDP-galactose, CMP-N-acetylneuraminic acid. At the same time a number of genes which encode enzymes known to be characteristic of non-human glycosylation reactions, will have to be deleted.

(4) Please replace the paragraph on **page 33, lines 7-24** (encompassing Example 1 and its title) with the following amended paragraph:

Example 1: Engineering of *P. pastoris* with α -1,2-Mannosidase to produce interferon.

An α -1,2-mannosidase is required for the trimming of $\text{Man}_8\text{GlcNAc}_2$ to yield $\text{Man}_5\text{GlcNAc}_2$, an essential intermediate for complex *N*-glycan formation. An *OCH1*

mutant of *P. pastoris* is engineered to express secreted human interferon- β under the control of an *aox* promoter. A DNA library is constructed by the in-frame ligation of the catalytic domain of human mannosidase IB (an α -1,2-mannosidase) with a sub-library including sequences encoding early Golgi localization peptides. The DNA library is then transformed into the host organism, resulting in a genetically mixed population wherein individual transformants each express interferon- β as well as a synthetic mannosidase gene from the library. Individual transformant colonies are cultured and the production of interferon is induced by addition of methanol. Under these conditions, over 90% of the secreted protein includes interferon- β . Supernatants are purified to remove salts and low-molecular weight contaminants by C₁₈ silica reversed-phase chromatography. Desired transformants expressing appropriately targeted, active α -1,2-mannosidase produce interferon- β including *N*-glycans of the structure Man₅GlcNAc₂, which has a reduced molecular mass compared to the interferon of the parent strain. The purified supernatants including interferon- β are analyzed by MALDI-TOF mass spectroscopy and colonies expressing the desired form of interferon- β are identified.

(5) Please replace the first two numbered listings of Table 7 at **page 39, lines 2-4**, with the following amended lines:

1. European Bioinformatics Institute (EBI) is a centre for research and services in bioinformatics
2. Swissprot database